Heat Shock Protein 20 Interacting With Phosphorylated Akt Reduces Doxorubicin-Triggered Oxidative Stress and Cardiotoxicity

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Abstract—Doxorubicin (DOX) is a widely used antitumor drug, but its application is limited because of its cardiotoxic side effects. Heat shock protein (Hsp)20 has been recently shown to protect cardiomyocytes against apoptosis, induced by ischemia/reperfusion injury or by prolonged β-agonist stimulation. However, it is not clear whether Hsp20 would exert similar protective effects against DOX-induced cardiac injury. Actually, DOX treatment was associated with downregulation of Hsp20 in the heart. To elucidate the role of Hsp20 in DOX-triggered cardiac toxicity, Hsp20 was first overexpressed ex vivo by adenovirus-mediated gene delivery. Increased Hsp20 levels conferred higher resistance to DOX-induced cell death, compared to green fluorescent protein control. Furthermore, cardiac-specific overexpression of Hsp20 in vivo significantly ameliorated acute DOX-triggered cardiomyocyte apoptosis and animal mortality. Hsp20 transgenic mice also showed improved cardiac function and prolonged survival after chronic administration of DOX. The mechanisms underlying these beneficial effects were associated with preserved Akt phosphorylation/activity and attenuation of DOX-induced oxidative stress. Coimmunoprecipitation studies revealed an interaction between Hsp20 and phosphorylated Akt. Accordingly, BAD phosphorylation was preserved, and cleaved caspase-3 was decreased in DOX-treated Hsp20 transgenic hearts, consistent with the antiapoptotic effects of Hsp20. Parallel ex vivo experiments showed that either infection with a dominant-negative Akt adenovirus or preincubation of cardiomyocytes with the phosphatidylinositol 3-kinase inhibitors significantly attenuated the protective effects of Hsp20. Taken together, our findings indicate that overexpression of Hsp20 inhibits DOX-triggered cardiac injury, and these beneficial effects appear to be dependent on Akt activation. Thus, Hsp20 may constitute a new therapeutic target in ameliorating the cardiotoxic effects of DOX treatment in cancer patients. (Circ Res. 2008;103:000-000.)

Key Words: apoptosis | cardiomyopathy | doxorubicin | heat-shock protein | Akt

D oxorubicin (DOX), an anthracycline antibiotic, is a highly effective chemotherapeutic agent used in the treatment of solid and hematopoietic tumors; however, a major limiting factor for the clinical use of DOX is its cumulative, irreversible cardiac toxicity.1–3 In fact, multiple intravenous DOX treatments over a period of several months result in the development of cardiomyopathy and congestive heart failure in humans.3 The precise cellular mechanisms responsible for this chronic cardiotoxicity of DOX remain enigmatic, but the antitumor activity of DOX is likely to be distinct from the mechanism of its cardiotoxicity.4–5 Accumulating evidence indicates that DOX-induced cardiomyopathy is mainly caused by increased oxidant production, which eventually leads to the apoptotic loss of cardiomyocytes.4,5,6 Therefore, we speculated that suppression of apoptosis may largely rescue DOX-triggered cardiotoxicity.

Heat shock proteins (Hsps) play important roles in cellular stress resistance and development of tolerance as an adaptive response after exposure to various stimuli.7 It has been found that thermal preconditioning effectively protected cardiomyocytes against DOX-induced apoptosis,8,9 whereas this protective effect was attenuated by knockdown of Hsp70 by antisense mRNA in cardiomyocytes,8 suggesting that Hsps may regulate DOX-triggered cardiac injury. Other studies further demonstrated that elevated Hsp27 and Hsp70 levels were associated with DOX resistance in tumor cells.10 What is more, it has been suggested that pharmacologically or physiologically induced Hsp60 and Hsp70 overexpression is involved in the cardioprotection against DOX.11,12 However, DOX-induced expression alterations of Hsps in the heart are currently not clear. Furthermore, it is still questioned whether heart-specific overexpression of a Hsp could protect against DOX-induced cardiotoxicity. Recently, Hsp20 has been shown to protect hearts against cardiac myocyte apoptosis, induced by ischemia/reperfusion injury in vivo,13 and elevated Hsp20 in the cardiomyocytes renders protection against...
isoproterenol-triggered apoptosis in vitro and in vivo. However, the potential benefits of Hsp20 action on DOX-induced cardiac injury and its underlying mechanism(s) are largely unknown.

Therefore, on the basis of our previous findings, we postulated that increased Hsp20 levels would alleviate DOX-triggered cardiotoxicity. Our results demonstrate that Hsp20 could protect against DOX-induced cardiomyocyte death in vitro and in vivo, and further prolong the mouse survival after either acute or chronic administration of DOX. Importantly, the mechanism underlying the cardioprotective effects of Hsp20 against DOX toxicity involves: (1) interaction of Hsp20 with phosphorylated Akt, which protects Akt against dephosphorylation by protein phosphatase (PP)1; and (2) attenuation of DOX-triggered oxidative stress.

Materials and Methods

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Animal Preparation

Generation of cardiac-specific overexpressed Hsp20 mice has been previously described. Male wild-type (WT) and transgenic (TG) mice, inbred on a FVB/N background, were studied at 8 to 10 weeks. DOX was administered by IP injection at one dose of 20 mg/kg or a weekly dose of 3 to 4 mg/kg to a cumulative amount of 20 mg/kg. Control mice received injections of saline to a comparable volume. All procedures were in accordance with institutional guidelines for animal research.

Additional Methods

An expanded Materials and Methods section containing details regarding drug treatment in vitro; generation of plasmid pcDNA3-Hsp20 and adenovirus vectors: Ad.Hsp20 and Ad.dnAkt; cardiomyocyte isolation and cell culture; cell viability and apoptosis assay; cardiac contraction measurements; cardiotoxicity assay; Western blot analysis; coimmunoprecipitation and in vitro Akt kinase activity assay; oxidative stress, and reactive oxygen species (ROS) assay is available in the online data supplement.

Results

Effects of DOX on the Expression of Major Hsps in the Murine Heart

To assess the regulatory roles of cardiac Hsps in response to DOX treatment, we examined the expression profiles of the six major Hsps (Hsp90, Hsp70, Hsp60, Hsp27, αB-crystallin, and Hsp20) at different time points, following DOX IP injection. Western blots and quantitative results (Figure 1) indicated that alterations in the levels of Hsp70 and Hsp60 exhibited a biphasic response: (1) they greatly increased at 1 hour after DOX injection, and returned to basal levels by 2 hours; (2) they increased again at 4 to 12 hours and significantly decreased below basal levels at 2 to 3 days after DOX treatment. There was no significant change in Hsp90 expression, whereas DOX increased Hsp27 content by 2-fold at 30 minutes, and this increase was maintained up to 3 days. The levels of αB-crystallin were transiently increased at 30 minutes to 1 hour and then returned to basal. Although a similar transient increase was also observed with Hsp20 expression, its levels decreased by 40% at 2 to 3 days after DOX injection. These results demonstrate that the expression pattern of various Hsps is altered in the heart on DOX insult, suggesting that these Hsps may be involved in DOX-induced cardiomyopathy via different mechanism(s).

DOX-Induced Cardiomyocyte Death and Apoptosis Is Suppressed by Increased Hsp20 Levels

Several studies have shown that some Hsps can act as negative regulators of DOX-triggered apoptotic and necrotic
cell death, such as Hsp10 and Hsp60, which have been found to modulate DOX-induced mitochondrial apoptosis signaling in neonatal cardiomyocytes. However, the underlying mechanisms are still remain to be clarified. To investigate whether increased Hsp20 levels could have an inhibitory effect on DOX-induced cardiomyocyte death, we first infected H9c2 cells with Ad.Hsp20 or Ad.GFP for 24 hours and subsequently subjected them to DOX treatment. After 24 hours of treatment with DOX at 0.5 μmol/L, 40% of the Ad.GFP-infected cells were not viable as observed by phase-contrast microscopy (Figure 2A) and measured by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Figure 2B). However, DOX-induced cell death was markedly diminished in the Ad.Hsp20-infected group (Figure 2A and 2B). The cytoprotective effect of Hsp20 was also observed in cells treated with different concentrations of DOX or infected with different concentrations of Ad.Hsp20, which was dependent on the level of Hsp20 overexpression (Figure 2C and 2D), suggesting that Hsp20 acts directly on cardiomyocytes to inhibit DOX-induced cell death.

To further confirm that Hsp20 acts as a survival factor in cardiomyocytes, ventricular myocytes were isolated from adult rat hearts and infected with Ad.GFP or Ad.Hsp20. The infection efficiency reached more than 95% after 24 hours (Figure 2E). These infected cells were subsequently subjected to 20 μmol/L DOX treatment for 24 hours. Both MTS and DNA fragmentation assays showed that ectopic overexpression of Hsp20 protected adult rat cardiomyocytes against DOX-triggered cell death and apoptosis (Figure 2E).

**Figure 2.** Effect of increased Hsp20 expression on cardiomyocyte survival after DOX treatment. A. Photomicrographs of Ad.Hsp20-infected or Ad.GFP-infected H9c2 cells (50 multiplicities of infection) were taken in the presence or absence of DOX (0.5 μmol/L) for 24 hours. B. Ad.Hsp20-infected H9c2 cells showed significantly higher cell viability than Ad.GFP-infected cells after treatment with DOX at 0.5, 1, or 5 μmol/L for 24 hours. C. Western blots showed increased Hsp20 expression on increased multiplicities of infection (MOI) of adenoviral vector. D. Protection of H9c2 cells against DOX was shown to be dependent on the Hsp20 expression levels. E. There was no morphological change between the empty vector Ad.GFP- and Ad.Hsp20-infected myocytes, and more than 95% adult rat cardio-myocytes were infected by recombinant adenovirus vector after 24 hours, as indicated by green fluorescent protein (GFP) fluorescence (magnification, ×200). Hsp20-overexpressed cardiomyocytes were resistant to DOX-induced cell death, as evidenced by decreased number of round-shaped cardiomyocytes (magnification, ×100), and MTS analysis, as well as DNA fragmentation assay, determined by a cell death detection ELISA kit. *P<0.05 vs control Ad.GFP. Similar results were observed in 3 additional, independent experiments.

**Decreased DOX-Induced Cardiomyocyte Injury and Apoptosis in Hsp20 TG Hearts**

To determine whether Hsp20 overexpression protects cardiomyocytes against DOX-induced apoptosis in vivo, TG mice overexpressing Hsp20 under the control of the cardiac-specific α-myosin heavy chain promoter were analyzed. TG hearts showed a 10-fold increase in total (transgenic and endogenous) Hsp20 content compared with WT mouse hearts. Eight-week-old WT and TG mice were subjected to 1 IP injection of 20 mg/kg DOX or saline as a control. At 4 days after the injection of DOX, the hearts of DOX-treated WT mice (Figure 3A), but not those of DOX-treated TG mice (Figure 3B), displayed focal cytoplasmic vacuolization, a hallmark of cell injury, which is consistent with previous reports. The percentage of TUNEL-positive nuclei in the
DOX-treated WT hearts was 2.5-fold higher than in DOX-treated TG hearts (Figure 3C). Accordingly, DNA fragmentation, measured by an ELISA method, was reduced by 30% in DOX-treated TG hearts, compared with controls (Figure 3D). These results indicate that overexpression of Hsp20 in vivo significantly decreased DOX-induced cardiac injury and apoptosis.

**Preserved Cardiac Function in Hsp20 TG Mice after Acute DOX Administration**

Recent studies have shown that very low levels of myocyte apoptosis (23 myocytes per $10^5$ nuclei of animal hearts; 80 to 250 myocytes per $10^5$ nuclei of human hearts) significantly affect cardiac function and survival.\(^1\)\(^9\) Given the marked disparity of cell injury and apoptosis in DOX-treated WT and Hsp20 TG hearts, we examined contractile function and animal survival in a separate series of experiments. Four days after the injection of 20 mg/kg DOX or saline, WT and TG mouse hearts were subjected to functional measurement by the Langendorff mode. As shown in Figure 4A through 4D, DOX treatment caused a significant decrease in the rates of contraction (+dP/dt) and relaxation (−dP/dt) in WT hearts, accompanied by a significantly increased left ventricular end-diastolic pressure (LVEDP) and reduced left ventricular developed pressure (LVDP). However, these functional parameters showed no significant difference in TG hearts (Figure 4A through 4D). In addition, whereas 6 of 11 WT mice died within 12 days after treatment with DOX, most TG mice (12 of 14) survived for up to 12 days after treatment (Figure 4E; $P<0.05$ versus WT). These findings indicate that overexpression of Hsp20 in heart reduced the extent of DOX-induced acute heart failure and prolonged survival.

**Improved Cardiac Function and Survival in Hsp20 TG Mice After Chronic DOX Administration**

Because patients typically receive lower levels of DOX given over many weeks,\(^2\)\(^3\) we further investigated whether cardiac-specific overexpression of Hsp20 could improve myocardial function and prolong survival in a chronic model of DOX-induced cardiotoxicity. To develop such a chronic model, an initial DOX dose of 4 mg/kg was selected, and mice received injections weekly for 5 weeks. The survival curve of the treated groups indicated that there was no significant difference between WTs and TGs; although all the treated WT mice died, 3 of 9 treated TG mice survived during the 10 weeks of follow-up after the last DOX injection (Figure 5A). However, in an additional chronic model, mice received 3 mg/kg weekly for 6 weeks, and we observed that all the treated TG mice survived during the 7 weeks of follow-up after the last DOX administration, whereas 5 of 12 treated WT mice died (Figure 5B; $P<0.05$ versus WT). To determine the alteration of myocardial function in this chronic model, 1 week after the last administration of DOX (3 mg/kg weekly for 6 weeks), we measured myocardial contractility by the Langendorff preparation to avoid the in vivo confounding effects, such as loading conditions and neurohormonal factors. As shown in Figure 5C, the rates of contraction (+dP/dt) and relaxation (−dP/dt) were significantly improved, accompanied by a reduced LVEDP and an increased LVDP in DOX-treated TG hearts, compared with DOX-treated WT hearts.

**Akt-BAD Signaling Pathway Is Preserved in DOX-Treated Hsp20 TG Hearts**

To elucidate the possible mechanism(s) of Hsp20 cardioprotection against DOX, we first assessed the expression levels of other major Hsps in acute DOX-treated TG hearts and controls. Western blots showed that overexpression of Hsp20 did not alter the expression levels of other major Hsps (Hsp90, Hsp70, Hsp60, Hsp27, and αB-crystallin) either in DOX-treated or saline-treated hearts, compared with WT hearts (Figure I in the online data supplement), suggesting that Hsp20 overexpression does not result in compensatory adaptation by other Hsps, and clearly indicates that the sole increase in Hsp20 levels can lead to protection against DOX-induced cardiotoxicity.
A number of studies have shown that 2 classes of cell growth and survival/death signaling pathways, the phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase (MAPK) pathways, play important roles in regulating cell survival.20,21 Our results showed that there were no differences in activation of MAPK signaling between WT hearts and TGs after acute DOX administration (supplemental Figure II). However, DOX-treated WT hearts revealed significantly decreased activation of Akt (generally referred to as Akt1 hereafter), as determined by immunoblotting for phosphorylation of either Thr308 or Ser473. Importantly, phosphorylation of Akt was preserved in DOX-treated TG hearts (Figure 6A), which was further supported by in vitro kinase activity assays, using glycogen synthase kinase (GSK)-3α/β as a substrate (Figure 6B). Surprisingly, we found that this immunocomplex of p-Ser473-Akt contained

![Graphs and images demonstrating cardiac function and survival curves post DOX treatment.]

- **Figure 4.** Overexpression of Hsp20 in vivo improves cardiac function after acute administration of DOX (20 mg/kg), as determined by the rates of contraction (+dP/dt) and relaxation (−dP/dt) (A and B), the LVEDP (C), and the LVDP (D). E, Hsp20 overexpression also prolongs the animal survival after acute treatment of DOX. *P<0.05, compared with vehicle or WT group (n=5 for function measurement and n=11 to 14 for animal survival observation).

- **Figure 5.** Overexpression of Hsp20 in vivo prolongs the animal survival (A, 4 mg/kg per week for 5 weeks; B, 3 mg/kg per week for 6 weeks) and improves cardiac function (C) after chronic administration of DOX. Cardiac function was measured by ex vivo Langendorff preparations at 1 week after the last IP injection of DOX (3 mg/kg weekly 6 times). *P<0.05, compared with WT group (n=9 to 12 for animal survival observation and n=4 to 5 for functional measurement).
**Figure 6.** Effects of Hsp20 on the expression of Akt/BAD/caspase-3 signaling cascades in the DOX-treated hearts. Hearts were excised on day 4 after acute administration of DOX (20 mg/kg), homogenized with lysis buffer, and subjected to Western blotting analysis. A, The phosphorylation levels of Akt at either S473 or T308 site were significantly decreased in WT hearts after DOX treatment, whereas there were no alterations in Hsp20 TG hearts. B, In vitro Akt kinase activity assay by using glutathione S-transferase–fused GSK-3α/β cross-tide corresponding to residues surrounding GSK-3α/β (Ser21/9) as a substrate. This immunocomplex was also probed with Ser473-Akt or Hsp20 antibody. The same amount of heart homogenate (1 mg/200 μL) was immunoprecipitated with Ser473-Akt antibody. C, Reciprocal coimmunoprecipitation with Hsp20 antibody revealed an increased association of Hsp20 with Ser473-Akt in DOX-treated TG hearts. Preimmunoprecipitated WT heart homogenate was used as positive control (+), and immunoprecipitate without Hsp20 antibody was used as negative control (−). D, The Akt activity was normalized by the amount of Akt protein in the HEK293 cell lysates. E, The expressions of cleaved Caspase-3 were analyzed by Western blotting. F, The expressions of PP1, PP2A, PP2B, and α-Actin were analyzed by Western blotting.
Hsp20 (Figure 6B). Furthermore, reciprocal coimmunoprecipitation with an Hsp20 antibody showed an increased association between Hsp20 and phosphorylated Akt in DOX-treated Hsp20 TG hearts (Figure 6C). To further examine whether Hsp20 interacts with nonphosphorylated isoform of Akt, we coinfected H9c2 cells with a nonphosphorylatable form of Akt1-AAA (Ad.dnAkt) and Ad.Hsp20. Whereas Ad.Hsp20/Ad.dnAkt-cell lysates revealed a significant increase in total Akt level (the most is exogenous nonphosphorylated Akt1), immunoprecipitation experiments with the Hsp20 antibody showed that Hsp20-association Akt levels were similar to those in the Ad.GFP control (Figure 6D), suggesting that Hsp20 may selectively interact with phosphorylated Akt. In addition, coimmunoprecipitation results indicated that Hsp20 was associated with not only Akt1 but also Akt2 (supplemental Figure III) in the murine heart.

The marked disparity of Akt activity in DOX-treated hearts, we examined its downstream targets to assess their potential role in DOX-induced cardiotoxicity. Akt has been shown to promote cell survival via its ability to phosphorylate BAD at Ser136. Accordingly, p53-BAD was markedly reduced in DOX-treated WT hearts but not in DOX-treated TG hearts (Figure 6E). However, another critical downstream element of the Akt cell survival pathway, GSK-3β phosphorylation (Ser21), was unaltered in either WT or TG hearts after DOX treatment (data not shown). These results suggest that p-BAD alteration could be attributed to the antiapoptotic effects of Hsp20. Also, an active form of caspase-3, cleaved caspase-3, increased oxidative stress. To determine whether increased Hsp20 expression might be accompanied by the induction of antioxidant pathways, which detoxify ROS, we next measured the activities of antioxidant enzymes and ROS levels in heart homogenates. The enzymatic activities of both superoxide dismutase and glutathione peroxidase were significantly reduced in DOX-treated hearts, compared with saline-treated samples. However, these decreases were less pronounced in TG hearts, relative to WTs (Figure 8A and 8B). Similarly, the ROS levels were markedly increased in WTs after DOX treatment, but overexpression of Hsp20 attenuated this increase (Figure 8C). Furthermore, increased Hsp20 expression could effectively suppress 2′,7′-dichlorofluorescein (DCF) fluorescence in DOX-stressed cardiomyocytes (H9c2) in a dose-dependent fashion (Figure 8D), consistent with its antiapoptotic effects (Figure 2D).

Discussion

It is well accepted that cardiomyocyte apoptosis could be a fundamental part of the myocardial process that initiates or aggravates heart failure. For example, conditional overexpression of active caspase-8 demonstrated that very low levels of myocyte apoptosis are sufficient to cause lethal dilated cardiomyopathy. In this report, we provide the first evidence that overexpression of Hsp20 in vivo and in vitro protects against DOX-induced cardiomyocyte apoptosis and necrosis, resulting in prevention of cardiac dysfunction and improvement of animal survival. These data suggest that Hsp20 may play a positive regulatory role in the treatment of DOX-induced cardiomyopathy. Of interest, for the first time, we demonstrate that Hsp20 interacts with phosphorylated Akt and prevents DOX-triggered Akt dephosphorylation by phosphatase PP1, suggesting that increased association of Hsp20-
DOX leads to upregulation of phosphatase PP1 in the heart.

Specific significance of phosphatase activities.

Saline controls. These differences may reflect cell/tissue-specific significance of phosphatase activities.

PP2A or PP2B were similar between DOX-treated hearts and saline controls. Although PP1 is also reported to associate with and dephosphorylate Akt, which consequently results in increased Hsp27 levels in cardiomyocytes were correlated with more resistance to DOX-triggered cell death, and, accordingly, DOX-induced cardiac dysfunction and animal mortality were also significantly decreased. However, these observations could not exclude protective roles of other Hsps, because overexpression of Hsp27 in the heart upregulated both Hsp32 and Hsp70 expression in response to DOX treatment. Our present study clearly shows that overexpression of Hsp27 in cardiomyocytes against DOX.

Previous studies indicate that DOX-induced cardiotoxicity is associated with decreased levels of Akt phosphorylation.29,30 There is a increasing list of cardioprotective reagents, such as neuregulin-1, erythropoietin, leukemia inhibitory factor, and insulin-like growth factor-1, which attenuate the occurrence of apoptosis (Figure 8E).

Akt. This is supported by decreased association of Hsp20–BAD. Furthermore, Hsp20 binds with phosphorylated Akt, which may occur by phosphorylation of the Bcl-x inhibitor BAD at Ser136. However, it was not clear why DOX treatment causes dephosphorylation of Akt, which consequently results in cardiac injury. In the present study, we report that administration of DOX upregulated the expression of PP1 in the heart, which dephosphorylated Akt and its downstream S136-BAD. Furthermore, Hsp20 binds with phosphorylated Akt, which may block the action of PP1 on dephosphorylation of Akt. This is supported by decreased association of Hsp20–p-Akt in DOX-treated WT hearts and no alteration in DOX-treated TGs, compared with the saline groups. Although PP2A has been identified as a mediator of Akt dephosphorylation in fibroblasts, smooth muscle, and osteosarcoma, PP1 is also reported to associate with and dephosphorylate Akt in breast cancer cells, consistent with our present data in cardiomyocytes. Indeed, the expression levels of either PP2A or PP2B were similar between DOX-treated hearts and saline controls. These differences may reflect cell/tissue-specific significance of phosphatase activities.

At present, it is not entirely clear how chronic treatment of DOX leads to upregulation of phosphatase PP1 in the heart.

Several studies have considered oxidative stress as a major mediator of DOX-induced cardiac gene dysregulation.29,31,38 Although it is reasonable that DOX inhibits cardiac gene expression by inhibition of DNA replication/transcription or by elevated degradation of protein, it should be noted that certain genes encoding proteins, besides the present PP1, have also been found upregulated in a mouse model of chronic DOX cardiotoxicity.38 We also noticed that PP1 levels were downregulated in Hsp20 TG hearts under both basal conditions and DOX treatment, and the possible mechanism is currently under investigation.
Hsps were similar in both Hsp20 TGs and WTs treated with DOX. Nevertheless, protective roles, similar to that of Hsp20 against DOX-triggered cardiotoxicity, may be also played by other Hsps in vivo, and future studies may clarify the specificity of this response to Hsp20 using inducible or conditional knockout models. Furthermore, it remains unclear how PP1 affects Akt activity and how DOX treatment upregulates PP1 expression in the murine hearts.

In conclusion, the present work demonstrates that overexpression of Hsp20 in the heart attenuates DOX-induced cardiac injury. The mechanism underlying its protection, at least partially, depends on: (1) maintenance of Akt signaling cascades (Akt/BAD/caspase-3); and (2) attenuation of DOX-triggered oxidative stress, leading to inhibition of DOX-induced cardiomyocyte death and apoptosis (Figure 8E). Consequently, myocardial function and animal survival are improved. Thus, targeted therapy to increase Hsp20 expression in the heart may hold promise in suppressing DOX-triggered cardiac toxicity.

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Disclosures
None.

References


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Methods and Materials

Cell Cultures

The embryonic rat heart-derived cell line H9c2 was obtained from the American Type Culture Collection. Adult ventricular cardiomyocytes were isolated from 2-month old male Sprague-Dawley rats (Harlan Laboratory), as previously described (1, 2). Briefly, rats were anesthetized with sodium pentobarbital (50mg/kg IP) and heparinized (10,000U/kg IP). The hearts were perfused with preheated modified Krebs-Henseleit buffer (KHB) (in mmol/L: NaCl: 118, KCl: 4.8, HEPES: 25, K2PO4: 1.25, MgSO4: 1.25, glucose: 11, taurine: 5 and BDM: 10, pH 7.4) for 5 minutes. Hearts were then perfused with an enzyme solution, which contained 0.7mg/mL collagenase type II (263U/mg), 0.2mg/mL hyaluronidase, 0.1% BSA and 25μM Ca2+, for 10 minutes. Subsequently, the Ca2+ concentration in the perfusion buffer was raised to 100μM, and perfusion continued for 5 additional minutes. Finally, ventricular tissue was excised, minced, pipette-dissociated, and filtered through a 240-μm screen. Cells were harvested and resuspended in 0.2mM, 0.3mM, 0.4mM, 0.5 and 1mM Ca2+-KHB with 1% BSA, each for 5 min (let the cells settle down by gravity). Finally, the cells were centrifuged briefly again, and resuspended in ACCT medium consisting of DMEM containing 2mg/mL BSA, 2mM L-carnitine, 5mM creatine, 5mM taurine, 100IU/mL penicillin, and 100ug/mL streptomycin. Cells were then counted and plated on laminin-coated (10 ug laminin per mL PBS for 1h) glass coverslips or dishes. After 1-2 hours, the dishes were infected with adenoviruses in diluted media, at a multiplicity of infection of 500, for 2 hours before addition of suitable volume of culture media. Transfection efficiency, determined by GFP gene expression in the cultured cardiac myocytes under fluorescence microscopy, was consistently >95% by this method.
Adenoviral Constructs

The dominant-negative mutant of Akt1 cDNA, which encodes Akt1 protein with a hemagglutinin tag at the amino terminus and three amino acid substitutions at lysine 179, threonine 308, and serine 473 to alanine (Akt1AAA) was generously provided by Dr. K. Walsh (Boston University School of Medicine). Adenovirus vector expressing Akt1-AAA (named as Ad.dnAkt) was constructed as described (3-5). The adenovirus vector carrying cardiac Hsp20 cDNA (Ad.Hsp20) was generated by using the AdEasy system (6). The viruses were replicated in 293 cells, purified by Virakit™ Adeno (Virapur, ILC) and the viral titers were determined by plaque assay in 293 cells.

Cell Viability and Apoptosis Assay

Cell viability assessment was performed with the CellTiter 96 AQueous One Solution Cell Proliferation Assay Kit (Promega), as the manufacturer’s instructions. For apoptosis analysis, DNA fragmentation was determined by an ELISA kit (Roche Applied Science, Indianapolis, IN). Cardiomyocytes were lysed by gently dispersing the pellet using a pipette tip that was cut back to prevent shearing of cells and release of nuclear DNA. The extract was then centrifuged at low speed and assayed according to the manufacturer’s instructions. Fold increase was obtained by dividing the measured absorbance of an experimental group by the absorbance of the positive control, provided in the kit.

Drug Treatment in vitro

Doxorubicin (DOX, Sigma Chemical Co.), the mitogen-activated protein kinase kinase (MEK1/2) inhibitor PD98059, p38 inhibitor SB203580, JNK inhibitor II, PI3-Kinase inhibitors
LY294002 and wortmannin, were purchased from Calbiochem (La Jolla, CA). Stock solutions were prepared in de-ionized water (doxorubicin) or DMSO (for inhibitors). Myocytes were seeded and infected with adenoviruses one day before treatment. Then various doses of DOX were added to the cells for various lengths of time. For experiments using combinations of doxorubicin and other inhibitors, cells were pre-incubated with the inhibitors PD98059 (20 μM), SB203580 (20 μM), JNK inhibitor II (40 μM), Ly294002 (10 μM) or wortmannin 100 nM for one hour before addition of doxorubicin. The same volumes of corresponding solvents were added to the controls.

Animal Preparation

Generation of cardiac-specific overexpressed Hsp20 mice has been previously described (7). Male wild-type (WT) and transgenic (TG) mice, inbred on a FVB/N background, were studied at 8 to 10 weeks. Both WT and TG mice were randomly assigned to either the control group or the DOX-treated group. DOX was administered by intraperitoneal (ip) injection at one dose of 20 mg/kg (100 μl) or a weekly dose of 3 - 4mg/kg to a cumulative amount of 20 mg/kg. Control mice received injections of saline to a comparable volume 100 μl. All procedures were in accordance with institutional guidelines for animal research.

Cardiac Contraction Measurements and Cardiotoxicity Assay

Cardiac function was assessed ex vivo at 4 days (for acute treatment) or 1 week (for chronic treatment) after the last DOX or vechicle injection by Langendorff preparation, as previously described (7). For heart morphometry, hearts were excised at 4 days after Dox injection, and fixed with 10% buffered formalin, embedded in paraffin and cut from the apex to the base. In situ
DNA fragmentation was assessed using the DeadEnd™ Fluorometric TUNEL system (Promega), followed by staining with a mouse anti-α-sarcomeric actin antibody (1:50 dilution, Sigma-Aldrich) and DAPI (Invitrogen) (8). The percentage of TUNEL-positive myocytes was determined by counting 10 random fields per section under a microscope (BX50, Olympus). DNA fragmentation was also determined by an ELISA kit (Roche Applied Science, Indianapolis, IN) with 200 µg heart homogenates, as described above.

Immunoblotting
Heart homogenates or cells were harvested in RIPA buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing 10 µg/ml aprotinin, leupeptin and 5 mM PMSF and incubated for 30 min on ice. Then equal amounts of protein (60-100µg) from each sample were resolved on a 10% or 12% SDS-polyacrylamide gel by electrophoresis. Binding of the primary antibody was detected by peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Amersham), and bands were quantified with densitometry. Rabbit anti-Hsp90 (1:500 dilution), rabbit anti-Hsp70 (1:1000 dilution), mouse anti-Hsp60 (1:4000 dilution), mouse anti-Hsp27 (1:1000 dilution) and rabbit anti-αB-crystallin (1:1000 dilution) (Affinity BioReagents), rabbit anti-p38, anti-JNK, anti-ERK and their phosphorylation antibodies, rabbit anti-Akt and its phosphorylation (pS473Akt, pT308Akt) antibodies, rabbit anti-GSK-3β and its phosphorylation (Ser9)(1:1000 dilution), rabbit anti-BAD and its phosphorylation (Ser136) (1:250 dilution) (Cell Signaling Technology, Inc.), mouse anti-PP1(E-9), rabbit anti-PP2A(FL-309) and mouse anti-PP2B-Aα (D-9)(1:250 dilution), mouse anti-Akt1(B-1), anti-Akt2 (F-7) and rabbit p-Akt1/2/3 (Ser 473)-R (1:200 dilution) ( Santa Cruz Bitotech.), as well as mouse Hsp20 antibodies (1:5000 dilution, Research Diagnostics Inc.) were used. Caspase-3 activation was
determined by immunoblotting with measurement of cleaved caspase-3 (Asp175) (rabbit anti-cleaved caspase-3, 1:500 dilution, Cell Signaling). α-actin (1:1000 dilution, Sigma) was probed in each membrane as a loading control.

**Akt Kinase Activity Assay**

*In vitro* Akt kinase activity assay were performed by Western blotting, using phosphor-GSK-3α/β (Ser21/9) antibody (1:250 dilution) (Akt Kinase Assay Kit, Cell Signaling Technology, Inc.). Briefly, 20 μl of immobilized phosphorylated-Akt (Ser473) antibody bead was added to 200 μl (1mg) of heart homogenate, and incubated with gentle rocking overnight at 4 ºC. The mixture of beads-sample was microcentrifuged at 13,000rpm for 30 seconds, and the pellet was washed with 500 μl of 1 × Cell Lysis buffer twice, followed by washing pellet with 500 μl of 1 × Kinase buffer twice. Suspend the pellet in 50 μl of 1 × Kinase buffer supplemented with 1 μl of 10mM ATP and 1 μg of GSK-3 fusion protein, incubating for 30 min at 30 ºC. Terminate reaction with 50 μl of 2 × SDS sample buffer. Heat the sample to 95-100 ºC for 2-5 min. Vortex, then centrifuge for 5 min at 13,000rpm. Load 30 μl of sample per well on 12%SDS-PAGE gel. Except that phosphor-GSK-3α/β (Ser21/9) antibody (1:250 dilution) was probed, Hsp20 and Ser-473 Akt antibodies were also probed in the each membrane.

**Co-immunoprecipitation**

For the co-immunoprecipitation experiments, protein lysates, extracted from mouse cardiac homogenates or cultured cardiomyocytes with 1 × cell lysis buffer (Cell Signaling, #9803) supplemented with 1mM PMSF and protease inhibitor cocktail (Sigma), were centrifuged for 30 min at 13000rpm at 4°C. The anti-Hsp20 or anti-Akt1 or anti-Akt2 antibody (4μg) was added to
1ml diluted cell lysates ((μg/μl), incubated overnight on a rotary wheel at 4 °C. Protein G PLUS-agarose beads (Santa Cruz Bitotech.) were added (1μg antibody/10 μl agarose beads) and incubated for an additional 1-2 h at 4 °C. The agarose beads were washed 6 times with the cell lysis buffer, soubilized in 2×SDS-sample buffer, boiled at 95 °C for 5 min, analyzed by SDS-PAGE and processed for immunoblotting.

**Oxidative Stress and ROS Assay**

Antioxidant enzymes were measured by Glutathione Peroxidase(GPx)Assay Kit and Superoxide Dismutase (SOD) Assay Kit II (Calbiochem). Reactive oxygen species (ROS) levels were determined by the fluorescence indicator DCFH, as described elsewhere (9, 10). When DCFH is added to the heart homogenate, ROS in the homogenate will lead to the oxidation of DCFH, producing the fluorescent product DCF. To rule out the potential artifact of ROS generation, the specificity of DCF fluorescence signal was confirmed by adding different doses of SOD, an antioxidant enzyme provided in the SOD Assay Kit II, into the DOX-treated WT heart homogenates (Online Figure IV). In the present study, 1μM DCFH was incubated with 1 ml of 200 μg heart homogenate for 1 h, and fluorescence was recorded using a fluorometer (1420 multilabel counter, PerkinElmer Life Science) equipped with a 96-well plate reader at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. For measurement of ROS formation in cardiomyocytes, H9c2 cells were grown in 96-well plates (10,000 cells/well) and transfected with various doses of pcDNA3-Hsp20 containing mouse cardiac Hsp20 cDNA or control pcDNA3.1 for 48 h, followed by addition of DOX (0.5μM) for 24 h (since our Ad.Hsp20 vector contains GFP expression cassette, which will interfere with the fluorescent product DCF, we cloned Hsp20 cDNA into the pcDNA3.1 (+) at the sites of Hind III and Xba I). Then these
treated cells were incubated with DCFH (10 μM) for 1h. Data were collected by fluorometer, as described above.

**Statistical Analysis**

All values are expressed as mean ± SEM. Statistical significance was determined with one-way ANOVA, followed by Duncan multiple range comparison test using Super ANOVA (Abacus Concepts, Inc). Survival curves after DOX injection were created using the Kaplan-Meier method and compared by a log-lank test. Differences were considered statistically significant at a value of P<0.05.

**References**


Online Figure I. Effects of Hsp20 on the expression of major Hsps in the DOX-treated hearts. Hearts were excised on day 4 after acute administration of DOX (20 mg/kg), homogenized with lysis buffer, and subjected to Western-blotting analysis. There were no differences in the expression of major Hsps between WT and Hsp20-TG hearts after treatment without or with DOX. (n=4).
Online Figure II. Effects of Hsp20 on the expression of MAPK pathway in the DOX-treated hearts. Hearts were excised on day 4 after acute administration of DOX (20 mg/kg), homogenized with lysis buffer, and subjected to Western-blotting analysis. There were no differences in the activation of MAPK pathway between WT and Hsp20-TG hearts after treatment without or with DOX (n=4).
Online Figure III. Reciprocal immunoprecipitation experiments using Akt1, Akt2 or Hsp20 antibodies showed Hsp20 not only interacted with Akt1, but also interacted with Akt2 in the murine heart. Pre-immunoprecipitated heart homogenate was used as positive control (+), immunoprecipitation without any antibodies (only added protein G agarose beads) was used as negative control (-). Antibodies Akt1 (B-1), Akt2 (F-7), and p-Akt1/2/3 (Ser473)-R were from Santa Cruz Biotech Inc (1:200 dilutions for immuno blotting). Hsp20 antibody was from RD1 (1:4000 dilutions). For immunoprecipitation, antibody Akt1 or Akt2 or Hsp20 (4 μg) was added to 1mg (1ml) pre-cleared heart homogenates (detailed in Methods).
Online Figure IV

Online Figure IV. Various doses of SOD were added to the 1 ml mixture of 1μM DCFH/200 μg DOX-treated WT heart homogenates, and incubation for 1 h at room temperature. Fluorescent intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.